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(54) Title: LIPID CARRIER COMPOSITIONS FOR IMPROVED DRUG RETENTION

(57) Abstract: Substantially cholesterol-free liposomes are provided which demonstrate improved drug retention *in vivo*. These liposomes may comprise: (a) at least 60 mol.% of a phospholipid comprising two saturated fatty acids, the acyl chain of each being the same or different, at least one of said acyl chains having more than 18 carbon atoms; (b) from about 2 to about 15 mol.% hydrophilic polymer-conjugated lipids; and (c) up to about 38 mol.% of one or more vesicle-forming lipids. Specific embodiments of this invention are liposomes encapsulating idarubicin or topotecan and demonstrating improved drug retention. Also provided is a method for determining whether retention of a particular drug may be improved by this invention.

LIPID CARRIER COMPOSITIONS FOR IMPROVED DRUG RETENTION

Technical Field

This invention is directed toward improving drug retention in lipid-based therapeutic carrier systems.

Background of the Invention

Liposomes and other lipid-based carrier systems have been extensively developed and analyzed for their ability to improve the therapeutic index of drugs by altering the pharmacokinetic and tissue distribution properties of drugs. This approach is aimed at reducing exposure of healthy tissues to therapeutic agents while increasing drug delivery to a diseased site.

Some drugs, and in particular, many anti-neoplastic drugs, are known to have a short half-life in the bloodstream such that their parenteral use is not optimized. The use of lipid-based carriers such as liposomes for site-specific delivery of such drugs via the bloodstream presents possible means to improve the use of such drugs. However, the use of liposomes for site-specific delivery is limited by the rate of clearance of liposomes from the blood, for example by cells of the Mononuclear phagocytic system (MPS). Furthermore, drugs encapsulated into liposomes are often not retained in the liposome after intravenous administration. In order for therapeutic effectiveness of liposome encapsulated drugs to be realized, such drugs must be effectively retained within a liposome after intravenous administration and the liposomes must have a sufficient circulation lifetime to permit the desired drug delivery.

It has long been established that incorporation of membrane rigidification agents such as cholesterol into a liposomal membrane enhances circulation lifetime of the liposome as well as retention of drugs within the liposome. Inclusion of cholesterol in liposomal membranes has been shown to reduce release of drug after intravenous administration (for example, see: United States Patents 4,756,910, 5,077,056, and 5,225,212; Kirby, C., *et al.* (1980) Biochem. J. 186:591-598; and, Ogihara-Umeda, I. and Kojima, S. (1989) Eur. J. Nucl. Med 15:617-7). Generally, cholesterol increases bilayer thickness and fluidity while decreasing membrane permeability, protein interactions, and lipoprotein destabilization of the liposome. Conventional approaches to

liposome formulation dictate inclusion of substantial amounts (e.g. 30-45 mol %) cholesterol or equivalent membrane rigidification agents (such as other sterols) into liposomes.

A further advancement in the liposome field was the discovery that polymers such as polyethylene glycol (PEG) grafted to the surface of a liposome increases the circulation lifetime of a liposome by decreasing liposomal/protein interactions. Unfortunately, the presence of PEG-conjugated lipids in the membrane can promote leakage of drugs from a liposome.

One avenue of liposome development that has become an exception to the conventional wisdom regarding use of cholesterol is the development of "thermosensitive" drug carriers (for example, see United States Patent 6,200,598; and, Gaber, M., *et al.* (1996) Int. J. Radiation Oncology Biol. Phys. 36:1177-1187). Thermosensitive liposomes are designed to have a phase transition temperature slightly above body temperature so that the liposomes remain in a gel state while in circulation but exceed the phase transition temperature upon application of heat to a patient's body or specific tissues. When heated, the liposome releases an encapsulated drug because the liposome bilayer becomes much more permeable above the transition temperature. Since cholesterol has the effect of broadening the phase transition temperature (inclusion of about 30 mol % or more cholesterol will usually eliminate phase transition entirely) thermosensitive liposomes are often made without cholesterol. Further, to have a phase transition temperature sufficiently close to normal human body temperature (e.g. 40-45°C), the lipid composition of the liposome is carefully tailored. A preferred lipid for use in thermal-sensitive liposomes is DPPC, which has an acyl chain length of 16 carbon atoms. Incorporation of any substantial amount of lipids having longer acyl chain lengths will raise the phase transition temperature of the liposome beyond the point of usefulness in thermosensitive applications. While circulation lifetime of a thermosensitive liposome may be enhanced by inclusion of PEG-conjugated lipids into the liposome just as in more conventional liposomes (see: United States Patent 5,843,473; Unezaki, S., *et al.* (1994) Pharm. Res. 11:1180-5; Maruyama, K., *et al.* (1993) Biochimica et Biophysica Acta 1149:209-206; Blume, G. and Cevc, G. [1] & [2] Biochimica et Biophysica Acta (1990)1029:91-97[1] & (1993) 1146:157-168[2]), thermosensitive liposomes exhibit poor drug retention *in vivo*.

Summary of Invention

This invention is based on the discovery that liposomes having phase transition temperatures above that which is useful for thermosensitive applications will better retain certain drugs, if the liposome is substantially free of cholesterol. In such cases, drug retention by the liposome is increased over an equivalent liposome formulation containing cholesterol. Appropriate circulation lifetime of such "cholesterol-free" liposomes may be achieved by incorporation of PEG-conjugated lipids.

The identity of drugs better retained by "cholesterol-free" liposomes in accordance with this invention is not predictable. Therefore, this invention provides methods for determining whether retention of a particular drug is enhanced by elimination of cholesterol from a liposome. This invention also provides improved drug retention in liposomes for specific drugs which previously exhibited poor retention in conventional cholesterol-containing liposomes. Also provided are novel cholesterol-free liposome formulations that are particularly suited for use in this invention.

This invention provides a method for determining whether retention of a drug in a liposome may be improved, comprising the steps of:

(a) preparing a liposome having a phase transition temperature greater than 45°C;

(b) preparing a liposome containing substantially the same lipids and in the same proportions as the liposome in (a) with at least 20 mol % cholesterol;

(c) encapsulating the drug into the liposomes of (a) and (b);

(d) administering the liposomes of (a) and (b) after encapsulation of the drug to the bloodstream of separate mammals;

(e) determining drug:lipid ratios in the blood of the mammals at at least one fixed time subsequent to administration; and

(f) comparing the ratios so determined for each mammal, wherein an increase in drug:lipid ratio in a mammal in which liposomes of (a) were administered as compared to drug:lipid ratio in a mammal in which liposomes of (b) were administered, is indicative of improvement in drug retention.

A particularly suitable non-human mammal for use in the aforementioned method is the mouse. Preferably, the liposome at (a) will exhibit a phase transition temperature of at least 50°C. Preferably, the amount of cholesterol in the liposome at (b) will be about

30 to about 50 mol %. Preferably, the drug:lipid ratios will be determined in step (e) at a series of intervals subsequent to administration with the comparison at (f) being of the ratios determined over the series of intervals.

Preferably, liposomes for use in step (a) of the preceding method will comprise at least 60 mol % of a phospholipid having two saturated fatty acids, the acyl chains of each having at least 18 carbon atoms. A preferred phospholipid with acyl chains of 18 carbon atoms each is DSPC. More preferably, liposomes for use in step (a) in the method above will have at least about 80, at least about 85, and even more preferably, at least 90 mol % of such a phospholipid. The remainder of the liposome may comprise one or more amphipathic lipids suitable for use in liposomes, but substantially no cholesterol. Preferably, such other lipids will include a hydrophilic polymer-conjugated lipid. Preferably, the amount of such polymer-conjugated lipids present in the liposome will be from about 2 to about 15 mol %.

Liposomes for use in the above method may be prepared using known and conventional techniques. Determination of phase transition temperatures, encapsulation of drug into liposomes (liposome loading), administration of liposomes, and determining drug:lipid ratios from blood may be carried out according to known and conventional techniques.

The above-described method may be used to select a liposome formulation to achieve optimal drug retention. Accordingly, this invention also provides a combination of a liposome and a drug wherein the liposome is a liposome as described above with respect to step (a) and the drug is an anti-neoplastic agent which exhibits greater retention in such a liposome, when the above-described method is performed. By "combination", it is meant that the drug is encapsulated in the liposome or is segregated but associated with the liposome (such as in a commercial package or kit comprising the liposome and the drug). Preferably, the liposome is one having the preferred characteristics of liposomes of step (a) as described above. Specific embodiments of this aspect of the invention include the combination of the above-described liposome with a idarubicin-compound or a camptothecin-compound.

This invention also provides novel liposomes which are particularly suitable for use in this invention, which liposomes comprise:

(a) at least 60 mol % of a phospholipid comprising two saturated fatty acids, the acyl chain of each being the same or different, at least one of said acyl chains having more than 18 carbon atoms;

(b) from about 2 to about 15 mol % hydrophilic polymer-conjugated lipid; and

(c) up to about 38 mol % of one or more vesicle-forming lipids, providing that the liposome contains substantially no cholesterol. Preferably, the liposome will contain essentially no cholesterol.

Preferred characteristics of the novel liposomes of this invention are those described above with respect to the liposome used in step (a) of the method, with the exception that the predominant phospholipid in the novel liposomes will have at least one acyl chain of greater than 18 carbon atoms.

This invention also provides the novel liposomes of this invention in combination with a drug and the use of such liposomes as a carrier for a drug encapsulated in the liposome. Such drugs include anti-neoplastic agents.

Brief Description of the Drawings

Figure 1: A graph showing encapsulation of idarubicin into DSPC:DSPE-PEG_{2000} (95:5 mol %) liposomes as a function of time at 37 (filled squares), 50 (open, inverted triangles) and 65 °C (filled circles).

Figure 2A: A histogram representing percent of lipid dose remaining in the blood for DSPC:cholesterol (55: 45 mol %) and DSPC:DSPE-PEG_{2000} (95:5 mol %) liposomes in CD-1 female mice after 1 hr.

Figure 2B: A histogram representing percent of idarubicin dose remaining in the blood after intravenous administration of DSPC:cholesterol (55:45 mol %) and DSPC:DSPE-PEG_{2000} (95:5 mol %) liposomes in CD-1 female mice after 1 hr.

Figure 2C: A histogram representing idarubicin:lipid ratio remaining in the blood for DSPC:cholesterol (55:45 mol %) and DSPC:DSPE-PEG_{2000} (95:5 mol %) liposomes in CD-1 female mice after 1 hr.

Figure 3A: A graph showing concentration of idarubicin (μmoles idarubicin/ mL plasma) recovered in the blood after intravenous injection of DSPC:cholesterol (55:45 mol %), DSPC:DSPE-PEG$_{2000}$ (95:5 mol %), DSPC: cholesterol: DSPE-PEG$_{2000}$ (50:45:5 mol %) liposomes and free idarubicin into Balb/c mice as a function of time represented by filled triangles, open circles, inverted filled triangles and open squares respectively.

Figure 3B: A graph showing concentration of lipid (μmoles total lipid/ mL plasma) recovered in the blood after intravenous injection of DSPC:cholesterol (55:45 mol %), DSPC:DSPE-PEG$_{2000}$ (95:5 mol %), DSPC:cholesterol:DSPE-PEG$_{2000}$ (50:45:5 mol %) liposomes into Balb/c mice as a function of time represented by filled triangles, open circles, inverted filled triangles respectively.

Figure 3C: A graph showing idarubicin:lipid ratio remaining in the blood after intravenous injection of DSPC:cholesterol (55:45 mol %), DSPC:DSPE-PEG$_{2000}$ (95:5 mol %) and DSPC:cholesterol:DSPE-PEG$_{2000}$ (50:45:5 mol %) liposomes into Balb/c mice as a function of time represented by represented by filled triangles, open circles, inverted filled triangles respectively.

Figure 4: A graph showing ratio of idarubicin:lipid after intravenous administration of idarubicin:lipid by injection in Balb/c mice as a function of time for liposomes consisting of DSPC:PEG2000 (95:5 mol %), DAPC:PEG2000 (95:5 mol %) and DBPC:PEG2000 (95:5 mol %) represented by filled circles, inverted open triangles and upward open triangles respectively.

Figure 5: A graph showing percent survival of P388 murine lymphocytic leukemia model as a function of time after intravenous injection of HBS (HEPES Buffered Saline) (filled circles), free idarubicin (open circles), idarubicin encapsulated in DSPC:DSPE-PEG2000 (95:5 mol%) liposomes (downward, filled triangles) and idarubicin encapsulated in DAPC:DSPE-PEG2000 (95:5 mol%) liposomes (downward, open triangles).

Figure 6: A graph showing topotecan:lipid ratio remaining in the blood after intravenous injection of DSPC:cholesterol (55:45 mol %) and DSPC:DSPE-PEG$_{2000}$ (95:5 mol %)

liposomes into Balb/c mice as a function of time represented by filled circles and open circles respectively.

Figure 7: A graph showing ratio of daunorubicin:lipid remaining in the blood after intravenous injection of Balb/c mice as a function of time for liposomes consisting of DSPC:cholesterol (CH) (55:45 mol %); DSPC:DSPE-PEG_{2000} (95:5 mol %); and, DSPC: CH:DSPE-PEG_{2000} (50:45:5 mol %) represented by filled circles, open circles, and inverted closed triangles, respectively.

Figure 8: A graph showing doxorubicin:lipid ratio remaining in the blood after intravenous injection of Balb/c mice as a function of time for liposomes consisting of DSPC:cholesterol (CHOL) (55:45 mol %); and, DSPC:DSPE-PEG_{2000} (95:5 mol %) represented by filled circles and open circles, respectively.

Detailed Description of the Invention

Throughout this specification, the following abbreviations have the indicated meaning. PEG: polyethylene glycol; PEG preceded or followed by a number: the number is the molecular weight of PEG in Daltons; PEG-lipid: polyethylene glycol-lipid conjugate; PE-PEG: polyethylene glycol-derivatized phosphatidylethanolamine; PA: phosphatidic acid; PE: phosphatidylethanolamine; PC: phosphatidylcholine; PI: phosphatidylinositol; DSPC: 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPE-PEG 2000 (or 2000 PEG-DSPE or PEG_{2000}-DSPE): 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[polyethylene glycol 2000]; DSPE-PEG 750 (or 750PEG-DSPE or PEG_{750}-DSPE): 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[polyethylene glycol 750]; DPPE-PEG2000: 1,2-dipalmaitoyl-*sn*-glycero-3-phosphoethanolamine-N-[polyethylene glycol 2000]; DAPC: 1,2-arachidoyl-*sn*-glycero-3-phosphocholine; DBPC: 1,2-dibehenoyl-*sn*-glycero-3-phosphocholine; CH or Chol: cholesterol; DPPC: 1,2-dipalmaitoyl-*sn*-glycero-3-phosphocholine; HEPES: N-[2-hydroxylethyl]-piperazine-N-[2-ethanesulfonic acid].

In this specification, the term "retention" with respect to a drug or other agent encapsulated in a liposome refers to retention of the drug in a liposome while the liposome is present in the bloodstream of a mammal. This term does not refer to a measure of drug

that may be loaded or incorporated into a liposome or the ability of a liposome to retain the drug in *ex vivo* conditions.

The term "cholesterol-free" as used herein with reference to a liposome means that a liposome is prepared in the absence of cholesterol, or that the liposome contains substantially no cholesterol, or that the liposome contains essentially no cholesterol. The term "substantially no cholesterol" allows for the presence of an amount of cholesterol that is insufficient to significantly alter the phase transition characteristics of the liposome (typically less than 20 mol % cholesterol). 20 mol % or more of cholesterol broadens the range of temperatures at which phase transition occurs, with phase transition disappearing at higher cholesterol levels. Preferably, a liposome having substantially no cholesterol will have about 15 or less and more preferably about 10 or less mol % cholesterol. The term "essentially no cholesterol" means about 5 or less mol %, preferably about 2 or less mol % and even more preferably about 1 or less mol % cholesterol. Most preferably, no cholesterol will be present or added when preparing "cholesterol-free" liposomes.

The term "phase transition temperature" is the temperature or range of temperatures at which a liposome changes from a gel state to a liquid crystalline state. A convenient method for measuring phase transition temperature is to monitor energy absorption while heating a preparation of liposomes and noting the temperature or range in temperatures at which there is an energy absorbance.

The term "liposome" as used herein means vesicles comprised of one or more concentrically ordered lipid bilayers encapsulating an aqueous phase. Formation of such vesicles requires the presence of "vesicle-forming lipids" which are amphipathic lipids capable of either forming or being incorporated into a bilayer structure. The latter term includes lipids that are capable of forming a bilayer by themselves or when in combination with another lipid or lipids. An amphipathic lipid is incorporated into a lipid bilayer by having its hydrophobic moiety in contact with the interior, hydrophobic region of the membrane bilayer and its polar head moiety oriented toward an outer, polar surface of the membrane. Hydrophilicity arises from the presence of functional groups such as hydroxyl, phosphato, carboxyl, sulfato, amino or sulfhydryl groups. Hydrophobicity results from the presence of a long chain of alaphatic hydrocarbon groups.

The term "hydrophilic polymer-lipid conjugate" refers to a vesicle-forming lipid covalently joined at its polar head moiety to a hydrophilic polymer, and is typically made

from a lipid that has a reactive functional group at the polar head moiety in order to attach the polymer. Suitable reactive functional groups are for example, amino, hydroxyl, carboxyl or formyl. The lipid may be any lipid described in the art for use in such conjugates other than cholesterol. Preferably, the lipid is a phospholipid such as PC, PE, PA or PI, having two acyl chains comprising between about 6 to about 24 carbon atoms in length with varying degrees of unsaturation. Most preferably, the lipid in the conjugate is a PE, preferably of the distearoyl form. The polymer is a biocompatible polymer characterized by a solubility in water that permits polymer chains to effectively extend away from a liposome surface with sufficient flexibility that produces uniform surface coverage of a liposome. Preferably, the polymer is a polyalkylether, including polymethylene glycol, polyhydroxy propylene glycol, polypropylene glycol, polylactic acid, polyglycolic acid, polyacrylic acid and copolymers thereof, as well as those disclosed in United States Patents 5,013,556 and 5,395,619. A preferred polymer is polyethylene glycol (PEG). Preferably the polymer has a molecular weight between about 1000 and 5000 daltons. The conjugate may be prepared to include a releasable lipid-polymer linkage such as a peptide, ester, or disulfide linkage. The conjugate may also include a targeting ligand. Mixtures of conjugates may be incorporated into liposomes for use in this invention.

The term "PEG-conjugated lipid" as used herein refers to the above-defined hydrophilic polymer-lipid conjugate in which the polymer is PEG.

The predominant vesicle-forming lipid in liposomes of this invention are responsible for achieving phase transition temperatures of greater than 45°C. Preferably, the lipid is a phospholipid such as PC, PE, PA or PE. The preferred phospholipid is PC. When selecting lipids, precautions should be taken since phase separation may occur if acyl chain lengths of these lipids differ by four or more methylene groups. Preferably the lipid will have two saturated fatty acids, the acyl chains of which being independently selected from the group consisting of stearoyl (18:0), nonadecanoyl (19:0), arachidoyl (20:0), heniecosanoyl (21:0), behenoyl (22:0), tricosanoyl (23:0), lingnoceroyl (24:0) and cerotoyl (26:0). In novel liposomes of this invention, at least one (and preferably both) of the acyl chains will be 19:0, or longer.

Liposomes of this invention may comprise amphipathic lipids in addition to those described above, but no substantial amount of cholesterol. Such lipids include

sphingomyelins, glycolipids, ceramides and phospholipids. Such lipids may include lipids having therapeutic agents, targeting agents, ligands, antibodies or other such components which are used in liposomes, either covalently or non-covalently bound to lipid components.

The terms "drug" and "therapeutic agent" as used herein refer to chemical moieties used in therapy and for which liposome-based drug delivery is desirable. The term "anti-neoplastic agent" as used herein refers to chemical moieties having an effect on the growth, proliferation, invasiveness or survival of neoplastic cells or tumours. Anti-neoplastic therapeutic agents include alkylating agents, antimetabolities, cytotoxic antibiotics and various plant alkaloids and their derivatives.

The term "idarubicin-compound" as used herein refers to idarubicin (e.g. 4-demethoxydaunorubicin) and related anthracyclines having the same core structure and hydrophobicity of idarubicin, including: daunorubicinol, 3'-hydroxyidarubicin, idarubicinol and WP619. This term includes salts of the aforementioned compounds. A preferred idarubicin-compound for use in this invention is idarubicin hydrochloride, sold for example under the trademark IDAMYCIN.

The term "camptothecin-compound" as used herein refers to camptothecin and derivatized forms of this plant alkaloid having topoisomerase inhibition activity, including topotecan, ironotecan, lurtotecan, 9-aminocamptothecin, 9-nitrocamptothecin and 10-hydroxycamptothecin, including salts thereof. A preferred camptothecin-compound is topotecan (hydrochloride salt) also known as hycamptamine.

Liposomes of the present invention or for use in the present invention may be generated by a variety of techniques including lipid film/hydration, reverse phase evaporation, detergent dialysis, freeze/thaw, homogenation, solvent dilution and extrusion procedures. Drugs may be encapsulated inside these liposomes by passive or active loading techniques known in the art. A particularly suitable encapsulation or liposome loading technique is pH gradient loading such as is described in the Examples herein. In the latter technique, liposomes are formed which encapsulate an aqueous phase of a selected pH. Hydrated liposomes are placed in an aqueous environment of a different pH selected to remove or minimize a charge on the drug or other agent to be encapsulated. Once the drug moves inside the liposome, the pH of the interior results in a charged drug

state, which prevents the drug from permeating the lipid bilayer, thereby entrapping the drug in the liposome.

Liposomes of this invention or for use in this invention may be from 50nm to about 1μm in diameter. However, preferred liposomes of this invention will be less than about 200 nm, preferably less than about 160 nm, and more preferably less than about 140 nm in diameter. 100-140 nm liposomes (cholesterol-free liposomes tend to be slightly larger than cholesterol containing ones) are employed in the Examples below. Liposomes are typically sized by extrusion through a filter (e.g. a polycarbonate filter) having pores or passages of the desired diameter.

Liposomes of this invention may be formulated for parenteral administration in a suitable carrier such as a sterile aqueous solution. The carrier may comprise excipients known to be tolerated by warm-blooded animals. When performing the assay method of this invention, a mammal such as a mouse will be injected with a liposome formulation and blood is removed from the mouse at fixed time intervals such as 1 or 4 or 24 hours post-administration. A convenient means for obtaining blood at a fixed time interval is by cardiac puncture. Following removal of whole blood, the plasma is isolated and subjected to suitable techniques for measuring the amount of lipid and drug present. For example, the lipid component may be radioactively labelled and the plasma subjected to liquid scintillation counting. The amount of drug may be determined by a spectraphotometric assay.

EXAMPLES

Source of Materials

DSPC and DSPE-PEG2000 were obtained from Northern Lipids. DAPC and DBPC were obtained from Avanti Polar Lipids. Cholesterol and Sephadex G50 was from Sigma Aldrich. 3H-cholesteryl hexadecyl ether (^{3}CHE) was from Perkin Elmer. Idarubicin hydrochloride (referred to below as idarubicin) was obtained from Pharmacia & UpJohn and topotecan from Smith Kline Beecham Pharma. Daunorubicin and doxorubicin were obtained from Rhone-Poulenc Rorer and Faulding Inc. respectively.

Example 1

Optimal uptake of idarubicin into cholesterol free liposomes occurs at 37°C

Solutions of DSPC and DSPE-PEG$_{2000}$ in chloroform were combined to give a 95: 5 molar ratio of DSPC: DSPE-PEG$_{2000}$ (80-100 μmoles total lipid) with 3 μCi/120 μmol lipid of ^{3}H-cholesteryl hexadecyl ether (^{3}CHE). The resulting mixture was dried under a stream of nitrogen gas and placed in a vacuum pump overnight. The samples were then hydrated with 300 mM citrate buffer pH 4.0, frozen (liquid nitrogen) and thawed (65 °C water bath) five times and subsequently passed through an extrusion apparatus (Lipex Biomembranes, Vancouver, BC) 10 times with 3 X 100 nm polycarbonate filters at 65 °C. Average liposome size was determined by quasi-elastic light scattering using a NICOMP 370 submicron particle sizer at a wavelength of 632.8 nm. The resulting liposomes were run down a Sephadex G50 column equilibrated with HBS (20 mM HEPES, 150 mM NaCl, pH 7.45) in order to create a transmembrane pH gradient by exchange of the exterior buffer. Resulting pH gradient liposomes were combined with idarubicin to give a final concentration of 5 mM lipid and 1 mM idarubicin (0.2:1 drug:lipid ratio) in a final volume of 1 mL adjusted with HBS. The resulting mixture was incubated at 37°C prior to assaying the amount of encapsulated idarubicin. At various time points, samples were fractionated on a 1 mL mini-Sephadex G-50 spin column to removed unencapsulated idarubicin. The voided fraction was assayed for liposomal lipid by scintillation counting. To measure levels of idarubicin, a defined volume of the eluant was adjusted to 100 μL followed by addition of 900 μL of 1% Triton X-100 to dissolve the liposomal membrane. The sample was heated until cloudy in appearance and the Abs$_{480}$ was measured after equilibration at room temperature. Concentrations of idarubicin were calculated by preparing a standard curve.

The above method was repeated using incubation temperatures of 50 and 65°C during drug loading.

Fig. 1 shows that optimal loading of idarubicin occurred at 37°C, while no loading was observed at 50 and 65°C possibly due to collapse of the pH gradient at higher temperatures.

Example 2

Recovery of idarubicin in plasma is increased in PEGylated, cholesterol-free liposomes relative to conventional, cholesterol-containing liposomes

pH gradient liposomes consisting of DSPC:cholesterol (55:45 mol %) and DSPC: DSPE-PEG_{2000} (95:5 mol %) were prepared according to the materials and methods of Example 1. The resulting liposomes were loaded as in Example 1 (drug:lipid ratio of 0.2 with 5 mM lipid and 1 mM idarubicin) at 37°C for 60 minutes. A total lipid dose of 3.3 μmoles (165 μmoles/kg) was administered to CD-1 female mice in a final volume of 200 μL immediately after preparation (within 1-2 hrs). Blood samples were removed at 1 hour after administration by cardiac puncture. Lipid concentration was determined by liquid scintillation counting. Idarubicin was extracted from plasma samples and quantified as follows:

A defined volume of plasma was adjusted to 200 μL with distilled water followed by addition of 600 μL of distilled water,100 μL of 10% SDS and 100 μL of 10 mM H_2SO_4. The resulting mixture was mixed and 2 mL of 1:1 isopropanol/chloroform was added followed by vortexing. The samples were frozen at -20°C overnight or -80°C for 1 hour to promote protein aggregation, brought to room temperature, vortexed and centrifuged at 3000 rpm for 10 minutes. The bottom organic layer was removed and assayed for fluorescence intensity at 500 nm as the excitation wavelength (2.5 nm bandpass) and 550 nm as an emission wavelength (10 nm bandpass) and using an absorbance wavelength of 480 nm.

The above method was repeated using DSPC:cholesterol (55:45 mol %), DSPC: cholesterol:DSPE-PEG_{2000} (50:45:5 mol %) and DSPC:DSPE-PEG_{2000} (95:5 mol %) pH gradient liposomes except blood samples were removed at 15 min, 30 min, 1, 2 and 4 hours after intravenous injection.

Fig. 2A, B and C show that although lipid concentrations after intravenous administration of DSPC:cholesterol (55:45 mol %) and DSPC:DSPE-PEG_{2000} (95:5 mol %) were comparable, idarubicin levels were 5 fold higher in the cholesterol-free, PEGylated formulations after 1 hr. This is further exemplified in Fig. **3A, B and C** at various time points after intravenous injection.

Example 3

Retention of drug in cholesterol-free liposomes increases with increasing acyl chain length

pH gradient liposomes consisting of DSPC:PEG2000 (95:5 mol %) DAPC (95:5 mol %) and DBPC (95:5 mol %) were prepared and loaded with idarubicin. Except during loading, concentrations of lipid and idarubicin were 16.5 mM and 2.2 mM respectively. Immediately following loading, (within 1-2 hrs) the liposomes were administered to Balb/C mice and blood samples were removed at 15 min, 30 min, 1, 2, 4 and 24 hours after intravenous injection and assayed for lipid and idarubicin concentrations as in Example 2.

Figure 4 shows that cholesterol-free liposomes exhibit enhanced retention of idarubicin as the length of the acyl chain is increased from 18 to 24 carbon atoms.

Example 4

Enhanced survival time upon administration of liposomal idarubicin

DSPC:DSPE-PEG2000 (95:5 mol%) and DAPC:DSPE-PEG2000 (95:5 mol%) liposomes were prepared and loaded with idarubicin as outlined in the materials and methods of Example 1 and Example 3 respectively.

P388/wt cells were maintained by passage in vivo (in the peritoneum) of BDF-1 female mice. Cells were only used for experiment between the 3rd and 20th passage. Cells were harvested 7 days post inoculation, diluted in Hepes Buffered Saline (HBS) to 2 x 10^6 cells/mL, and 0.5 mL was injected intraperitoneally into BDF-1 mice. Two days after tumor cell inoculation, BDF1 female mice were administered by intravenous administration one of the following: HBS; idarubicin (1 mg/kg); DSPC:DSPE-PEG2000 (95:5 mol%) and DAPC:DSPE-PEG2000 (95:5 mol%) liposomes (1 mg/kg) loaded with idarubicin. Percent survival was calculated based on 4 mice per group.

Figure 5 shows that percent survival was increased for idarubicin encapsulated in cholesterol-free DSPC and DAPC liposomes relative to administration of free drug.

Example 5

Recovery of topotecan in plasma is increased in PEGylated, cholesterol-free liposomes relative to conventional, cholesterol-containing liposomes

DSPC/cholesterol (55:45 mol %) and DSPC/DSPE-PEG$_{2000}$ (95:5 mol%) liposomes were prepared as in Example 1 except the dried lipid films were hydrated in 300 mM manganese sulfate and the external buffer was exchanged with 300 mM sucrose, 30 mM EDTA, and 20 mM HEPES pH 7.4. Topotecan was encapsulated into the liposomes using an ionophore-mediated proton gradient. The divalent cation ionophore A23187 (1 μg ionophore/ μmol lipid) was first added to the liposomes and the mixture was incubated at 60°C for 15 min. to facilitate incorporation of the ionophore into the bilayer. Subsequently, topotecan was added at a drug:lipid ratio of 0.08:1 and the mixture was incubated for 1 hr at 60° C. Unencapsulated topotecan and A23187 were removed from the preparation by dialyzing the sample at room temperature for 24 hrs against 100 volumes of 300 mM sucrose.

Liposomal topotecan (100 mg/kg lipid, 8 mg/kg topotecan) was injected into female Balb/C mice and the plasma elimination of both the lipid carrier and the drug were determined over a 24 hr. time course. Lipid concentrations remaining in the blood were measured by liquid scintillation counting. Topotecan was quantified by fluorescence spectroscopy. Plasma proteins were precipitated by the addition of 200 μL of methanol to 50 μL of plasma followed by centrifugation for 10 min. at 3500 rpm. The supernatant was removed and assayed for fluorescence intensity using a Perkin-Elmer LS50 fluorescence spectrophotometer set at an excitation wavelength of 380 nm (2.5 nm slit width) and emission wavelength of 518 nm (2.5 nm slit width).

EXAMPLE 6

The methods employed in the preceding examples were performed to determine drug retention in cholesterol containing and cholesterol-free liposomes in cases where the drug is either daunorubicin (Fig. 7) or doxorubicin (Fig. 8). The latter drugs are anthracycline anti-neoplastic agents which are similar in structure to idarubicin. In contrast to the results described above for idarubicin and topotecan, retention of

daunorubicin and doxorubicin is decreased in cholesterol-free liposomes as compared to conventional liposomes containing cholesterol (with or without PEG-conjugated lipids).

Comparison of drug retention for the various drugs described above in cholesterol containing and cholesterol-free liposomes may be conveniently expressed by calculating the "area-under-the-curve" (AUC) of the graphs which plot drug:lipid ratios as a function of time. The improvement in AUC over 0-4 hours post-administration that was observed in DSPC:DSPE-PEG liposomes as compared to DSPC:CHOL liposomes was +315% for idarubicin, +55% for topotecan, and -67% for daunorubicin. Thus, this invention can provide significant improvements in drug retention (e.g. greater than 50%) for some drugs.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of skill in the art in light of the teachings of this invention that changes and modification may be made thereto without departing from the spirit or scope of the appended claims. All patents, patent applications and publications referred to herein are hereby incorporated by reference.

WE CLAIM:

1. A liposome comprising:

(a) at least 60 mol % of one or more phospholipids, each phospholipid comprising two saturated fatty acids, the acyl chain of each being the same or different, at least one of said acyl chains having more than 18 carbon atoms;

(b) from about 2 to about 15 mol % of one or more hydrophilic polymer-conjugated lipids; and

(c) up to about 38 mol % of one or more vesicle-forming lipids, providing that the liposome contains substantially no cholesterol.

2. The liposome of claim 1, containing essentially no cholesterol.

3. The liposome of claim 1, containing about 1 or less mol % cholesterol.

4. The liposome of claim 1, 2, or 3, wherein a hydrophilic polymer-conjugated lipid is a PEG-lipid.

5. The liposome of claim 4, wherein PEG in the PEG-lipid has a molecular weight from about 1000 to about 5000 daltons.

6. The liposome of claim 4 or 5, comprising from about 5 to about 10 mol % PEG-lipid.

7. The liposome of any one of claims 1-6, wherein the one or more phospholipids are selected from the group consisting of: PC, PE, PA and PE.

8. The liposome of any one of claims 1-6, wherein one or more of the phospholipids includes PC.

9. The liposome of any one of claims 1-8, wherein the acyl chains are independently selected from the group consisting of: stearoyl (18:0), nonadecanoyl (19:0), arachidoyl (20:0), heniecosanoyl (21:0), behenoyl (22:0), tricosanoyl (23:0), lingnoceroyl (24:0) and

cerotoyl (26:0), providing that if one of said chains on a phospholipid is 18:0, a second of said chains on the phospholipid is not.

10. The liposome of any one of claims 1-9, wherein each acyl chain has more than 18 carbon atoms.

11. The liposome of any one of claims 1-10, wherein the acyl chains are the same.

12. The liposome of any one of claims 1-11, wherein the liposome comprises at least about 80 mol % of the one or more phospholipids.

13. The liposome of any one of claims 1-11, wherein the liposome comprises at least about 85 mol % of the one or more phospholipids.

14. The liposome of any one of claims 1-11, wherein the liposome comprises about 90 mol % of the one or more phospholipids.

15. The liposome of any one of claims 1-11, wherein the liposome comprises about 95 mol % of the one or more phospholipids.

16. The liposome of any one of claims 1-15, wherein the phospholipid is DSPC.

17. The liposome of any one of claims 1-15, wherein the phospholipid is DAPC.

18. The liposome of any one of claims 1-15, wherein the phospholipid is DBPC.

19. The liposome of any one of claims 1-16, having a phase transition temperature of about 50°C or more.

20. The liposome of any one of claims 1-17, further comprising a drug encapsulated in the liposome.

21. The liposome of claim 20, wherein the drug is an anti-neoplastic agent.

22. The liposome of claim 21, wherein the anti-neoplastic agent is an idarubicin-compound or a camptothecin-compound.

23. The liposome of claim 21, wherein the anti-neoplastic agent is idarubicin or a salt thereof.

24. The liposome of claim 21, wherein the anti-neoplastic agent is topotecan.

25. A method for determining whether retention of a drug in a liposome may be improved, comprising the steps of:

(a) preparing a liposome having a phase transition temperature greater than 45°C;

(b) preparing a liposome containing substantially the same lipids and in the same proportions as the liposome in (a) with at least 20 mol % cholesterol;

(c) encapsulating the drug into the liposomes of (a) and (b);

(d) administering the liposomes of (a) and (b) after encapsulation of the drug to the bloodstream of separate mammals;

(e) determining drug:lipid ratios in the blood of the mammals at at least one fixed time subsequent to administration; and

(f) comparing the ratios so determined for each mammal, wherein an increase in drug:lipid ratio in a mammal in which liposomes of (a) were administered as compared to drug:lipid ratio in a mammal in which liposomes of (b) were administered, is indicative of improvement in drug retention.

26. The method of claim 25, wherein the liposome of (a) has a phase transition temperature of about 50°C or more.

27. The method of claim 25 or 26, wherein the amount of cholesterol in the liposome of (b) is from about 30 to about 50 mol %.

28. The method of any one of claims 24-27, wherein lipids in the liposomes of (a) and (b) are labelled to facilitate determining at (e).

29. The method of any one of claims 24-28, wherein the liposome of (a) comprises at least 60 mol % of one or more phospholipids having two saturated fatty acids, the acyl chains of each having at least 18 carbon atoms and the liposome comprises substantially no cholesterol.

30. The method of claim 29, wherein the liposome of (a) comprises essentially no cholesterol.

31. The method of claim 29 or 30, wherein the liposome of (a) comprises at least about 80 mol % of the one or more phospholipids.

32. The method of claim 29 or 30, wherein the liposome of (a) comprises at least about 85 mol % of the one or more phospholipids.

33. The method of claim 29 or 30, wherein the liposome of (a) comprises at least about 90 mol % of the one or more phospholipids.

34. The method of claim 29 or 30, wherein the liposome of (a) comprises at least about 95 mol % of the one or more phospholipids.

35. The method of any one of claims 24-28, wherein the liposome of (a) is a liposome according to any one of claims 1-19.

36. The method of any one of claims 24-35, wherein the comparing performed at (f) is indicative of improvement in drug retention and the method further comprises encapsulating the drug in a liposome of (a).

37. The method of claim 36, wherein the drug is an anti-neoplastic agent.

38. A liposome encapsulating an anti-neoplastic agent, wherein the liposome is a liposome showing improved drug retention as determined by the method of any one of claims 24-35.

39. The liposome of claim 38, wherein the anti-neoplastic agent is an idarubicin-compound or a camptothecin-compound.

40. The liposome of claim 38, wherein the anti-neoplastic agent is idarubicin or a salt thereof.

41. The liposome of claim 38, wherein the anti-neoplastic agent is topotecan or a salt thereof.

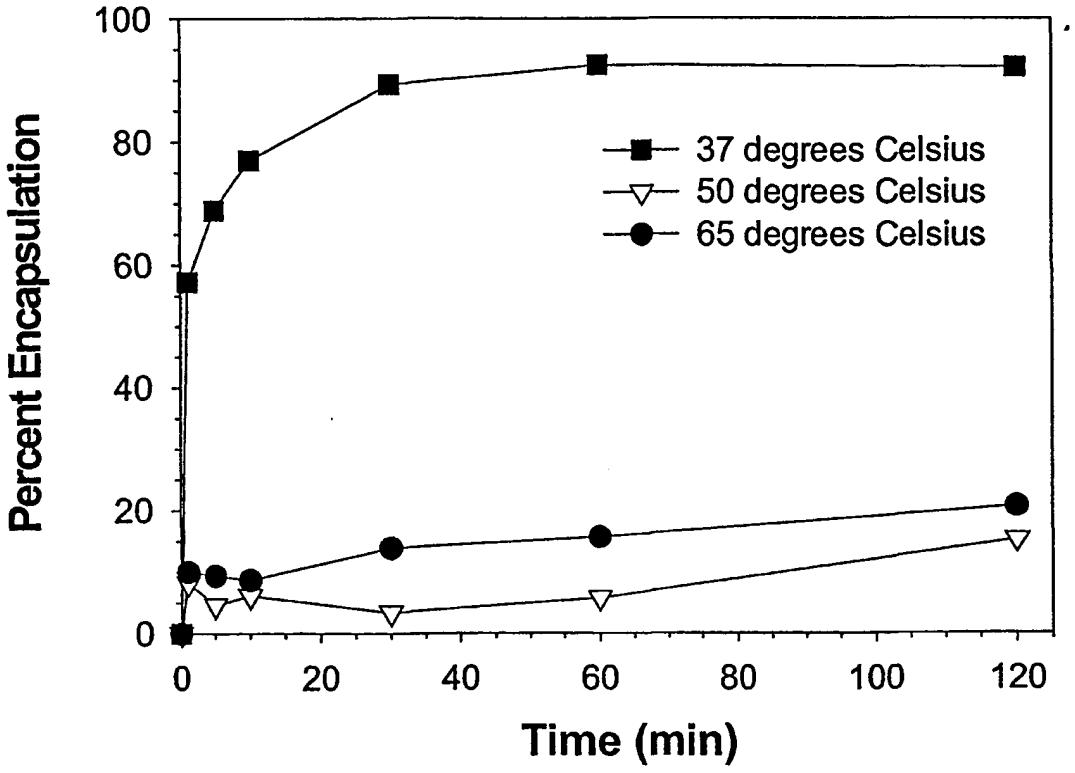
Percent Encapsulation
100
80
60
40
20
0
0
20
40
60
80
100
120
Time (min)
37 degrees Celsius
50 degrees Celsius
65 degrees Celsius

FIGURE 1

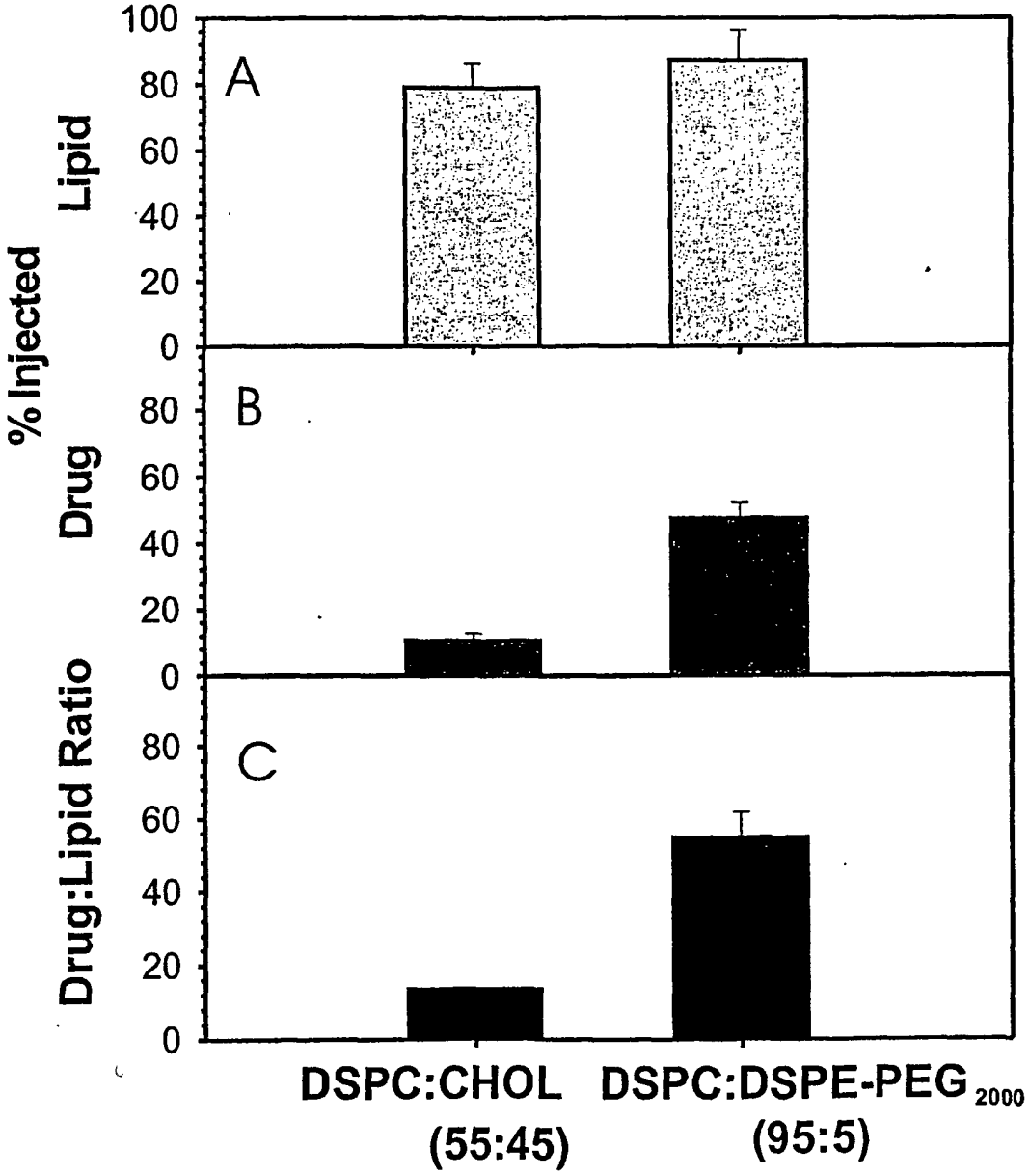
% Injected
Lipid
Drug
Drug:Lipid Ratio
A
B
C
100
80
60
40
20
0
DSPC:CHOL
(55:45)
DSPC:DSPE-PEG 2000
(95:5)

FIGURE 2

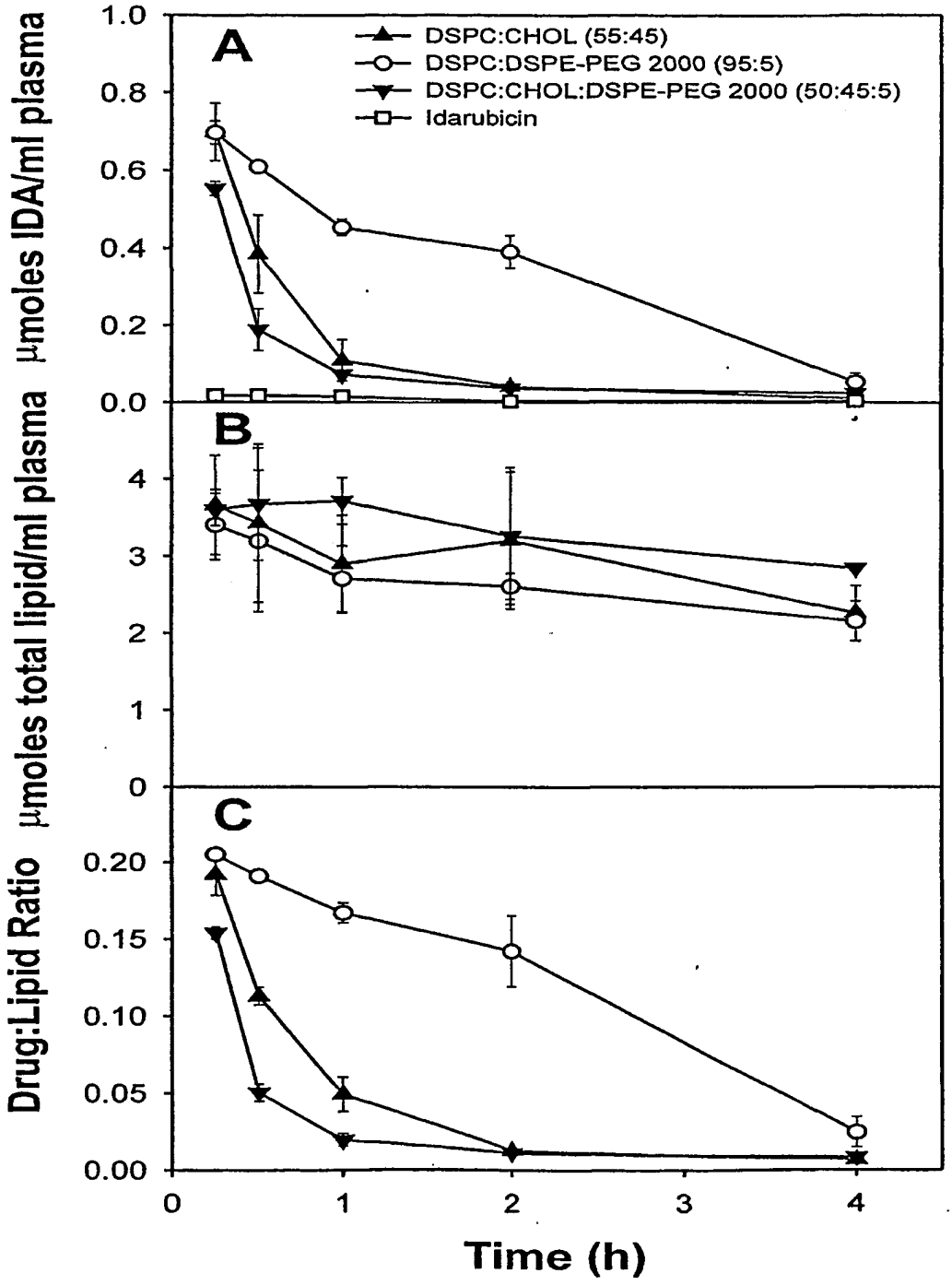
A
B
C
DSPC:CHOL (55:45)
DSPC:DSPE-PEG 2000 (95:5)
DSPC:CHOL:DSPE-PEG 2000 (50:45:5)
Idarubicin
µmoles IDA/ml plasma
µmoles total lipid/ml plasma
Drug:Lipid Ratio
Time (h)
1.0
0.8
0.6
0.4
0.2
0.0
4
3
2
1
0
0.20
0.15
0.10
0.05
0.00
0
1
2
3
4

FIGURE 3

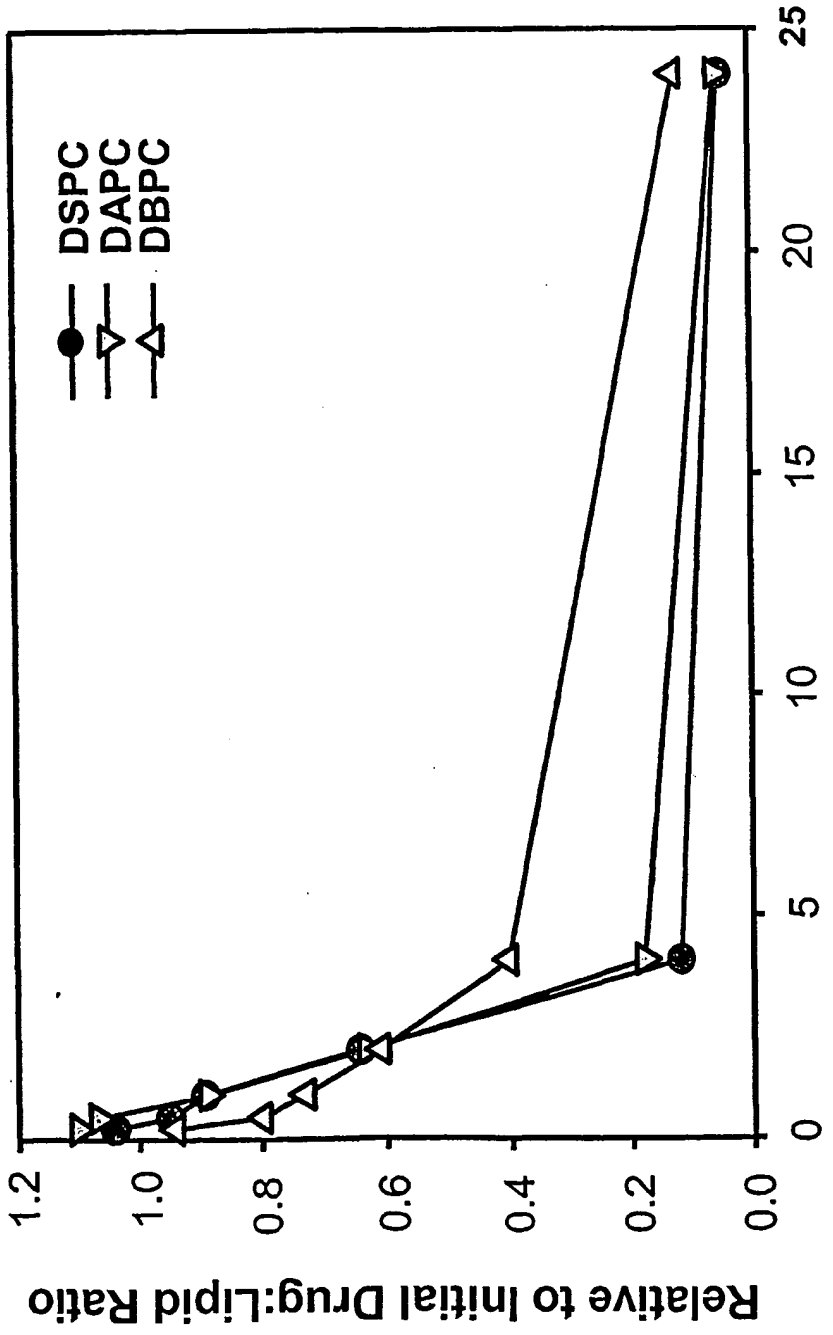
DSPC
DAPC
DBPC
Relative to Initial Drug:Lipid Ratio
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
5
10
15
20
25
Time (h)

FIGURE 4

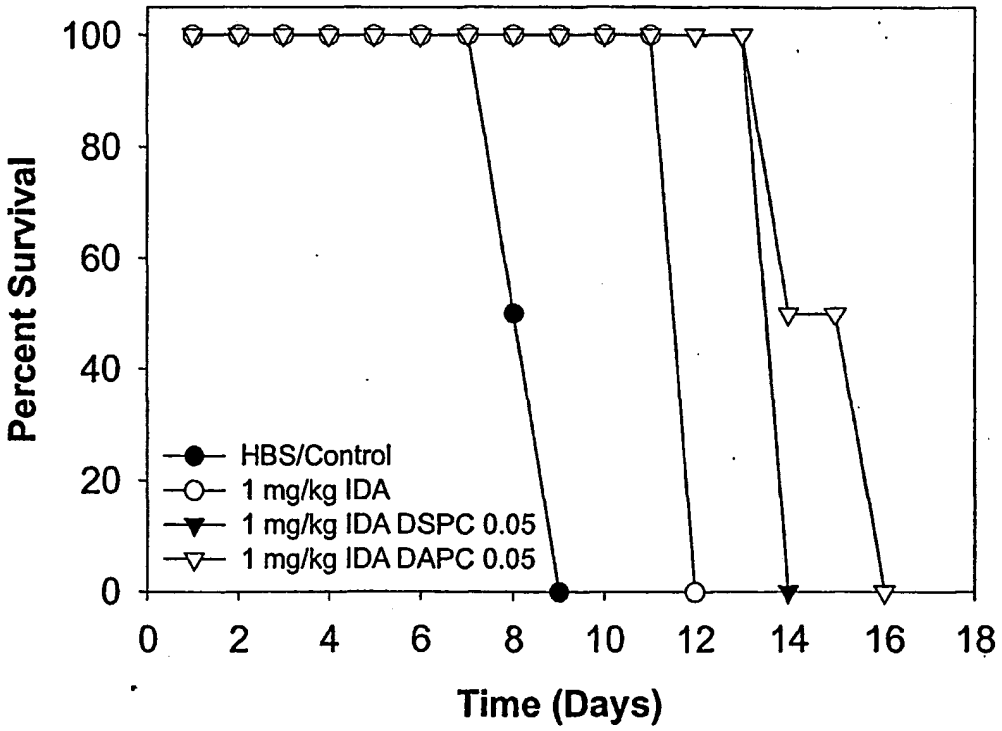
Percent Survival
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
Time (Days)
HBS/Control
1 mg/kg IDA
1 mg/kg IDA DSPC 0.05
1 mg/kg IDA DAPC 0.05

FIGURE 5

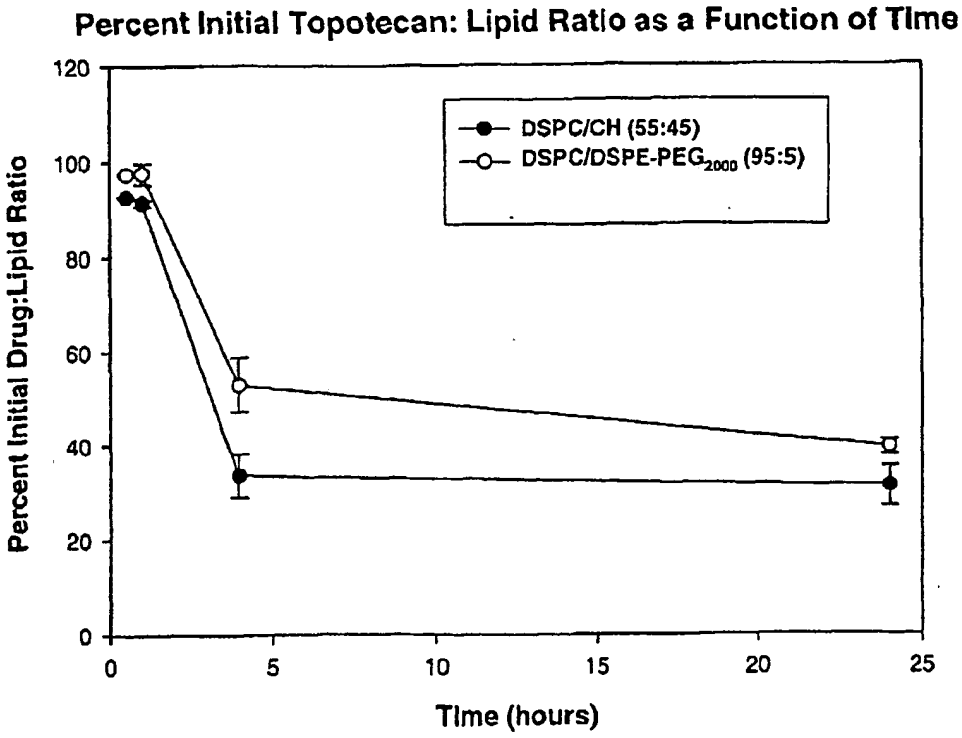
Percent Initial Topotecan: Lipid Ratio as a Function of Time
Percent Initial Drug:Lipid Ratio
120
100
80
60
40
20
0
0
5
10
15
20
25
Time (hours)
DSPC/CH (55:45)
DSPC/DSPE-PEG2000 (95:5)

FIGURE 6

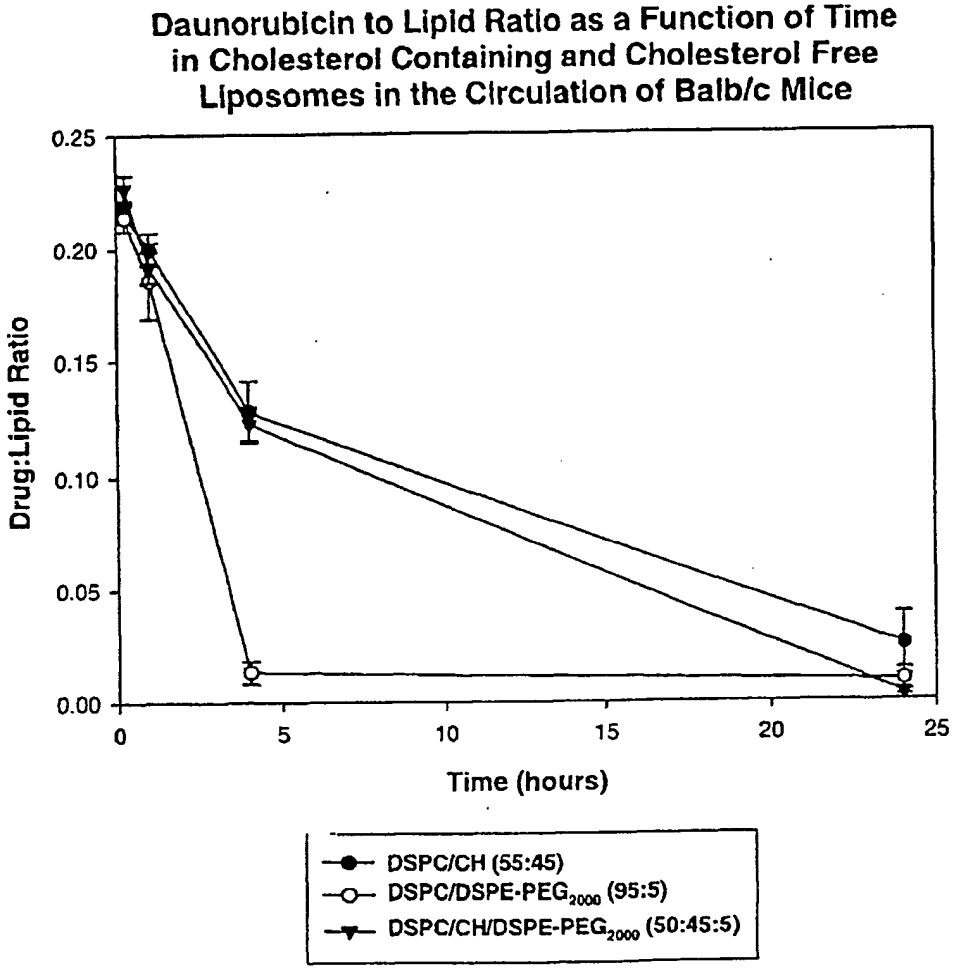
Daunorubicin to Lipid Ratio as a Function of Time in Cholesterol Containing and Cholesterol Free Liposomes in the Circulation of Balb/c Mice
Drug:Lipid Ratio
0.25
0.20
0.15
0.10
0.05
0.00
0
5
10
15
20
25
Time (hours)
DSPC/CH (55:45)
DSPC/DSPE-PEG2000 (95:5)
DSPC/CH/DSPE-PEG2000 (50:45:5)

FIGURE 7

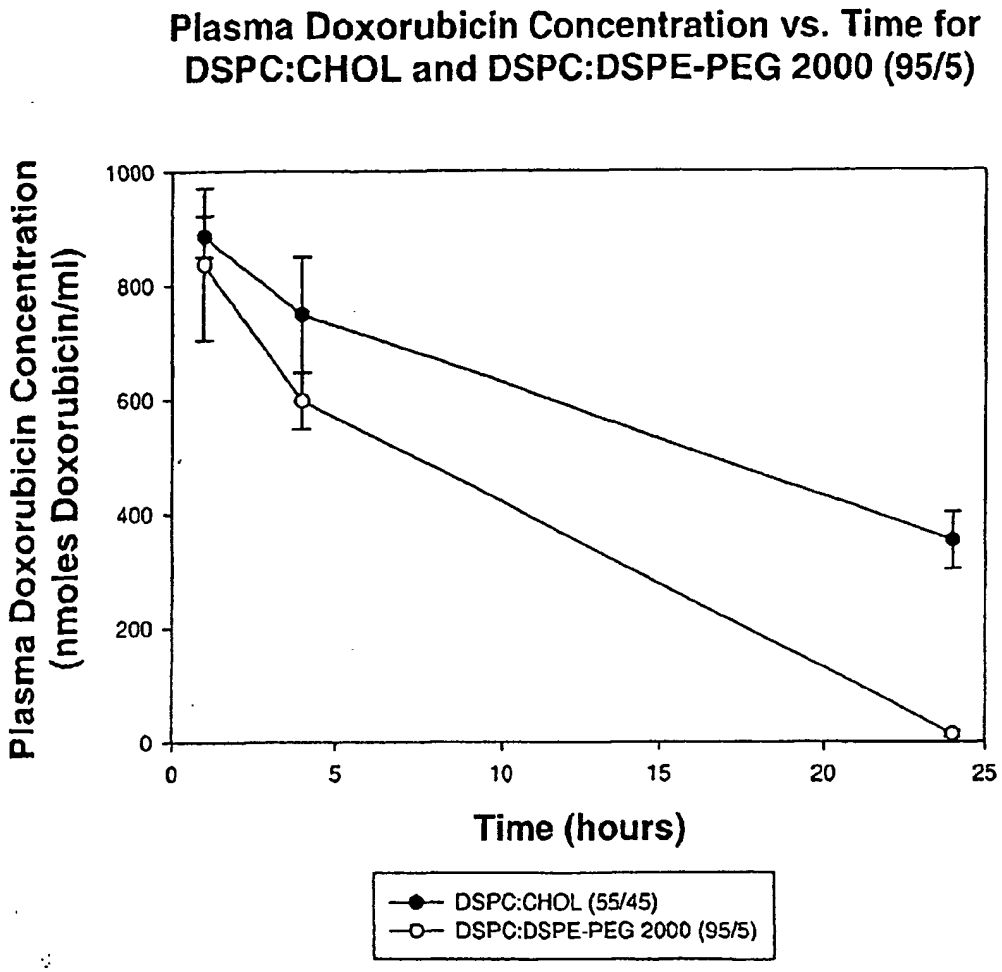
Plasma Doxorubicin Concentration vs. Time for DSPC:CHOL and DSPC:DSPE-PEG 2000 (95/5)
Plasma Doxorubicin Concentration (nmoles Doxorubicin/ml)
1000
800
600
400
200
0
0
5
10
15
20
25
Time (hours)
DSPC:CHOL (55/45)
DSPC:DSPE-PEG 2000 (95/5)

FIGURE 8